

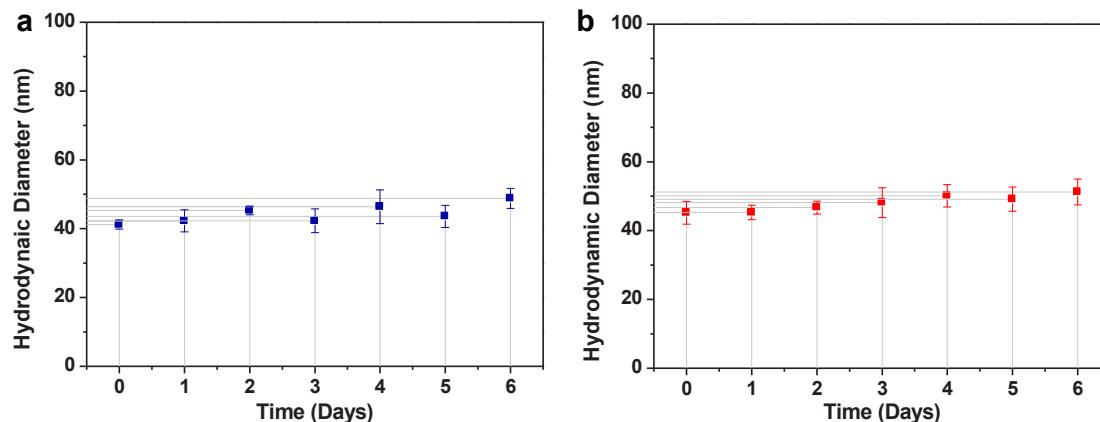
Semiconducting polymer nanoparticles as photoacoustic molecular imaging probes in living mice

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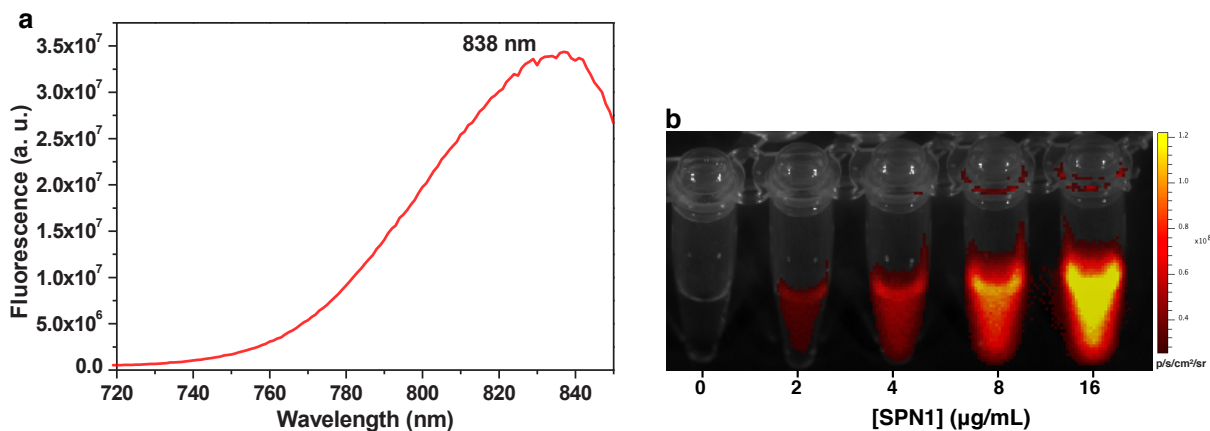
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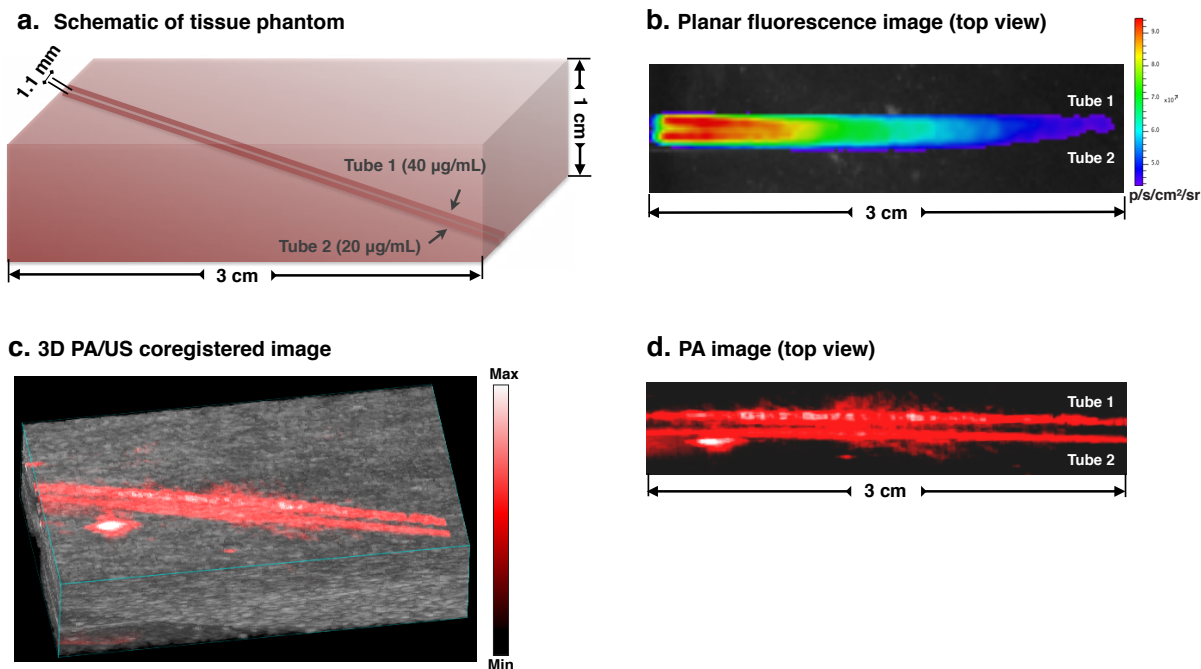
1. Supplementary Figures



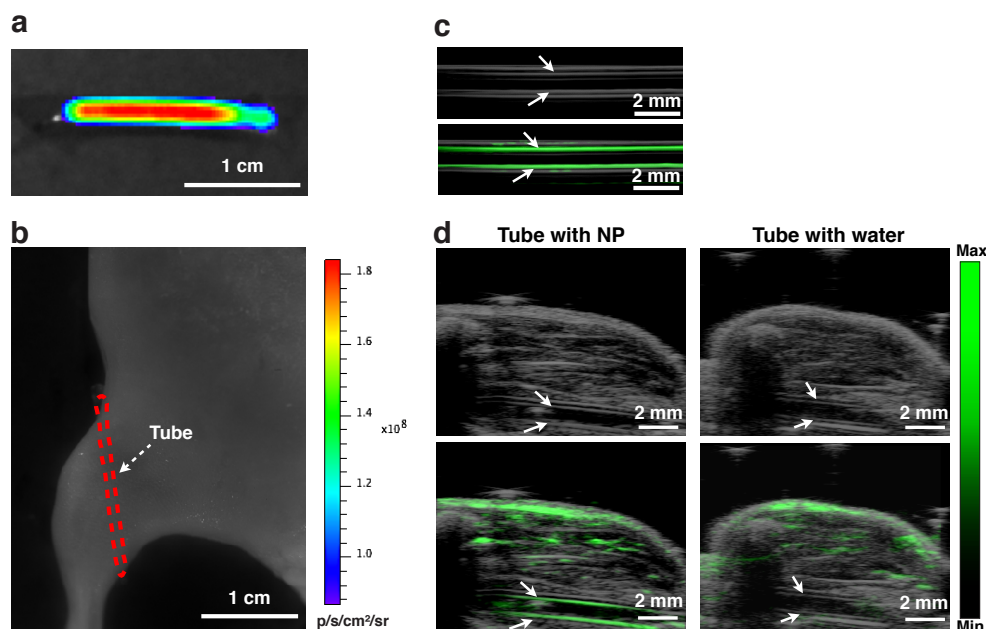
Supplementary Fig. S1: Stability study of SPN1 (a) and SPN2 (b) in 1×PBS (pH = 7.4) over 6 days. Error bars represent standard deviation of three separate measurements.



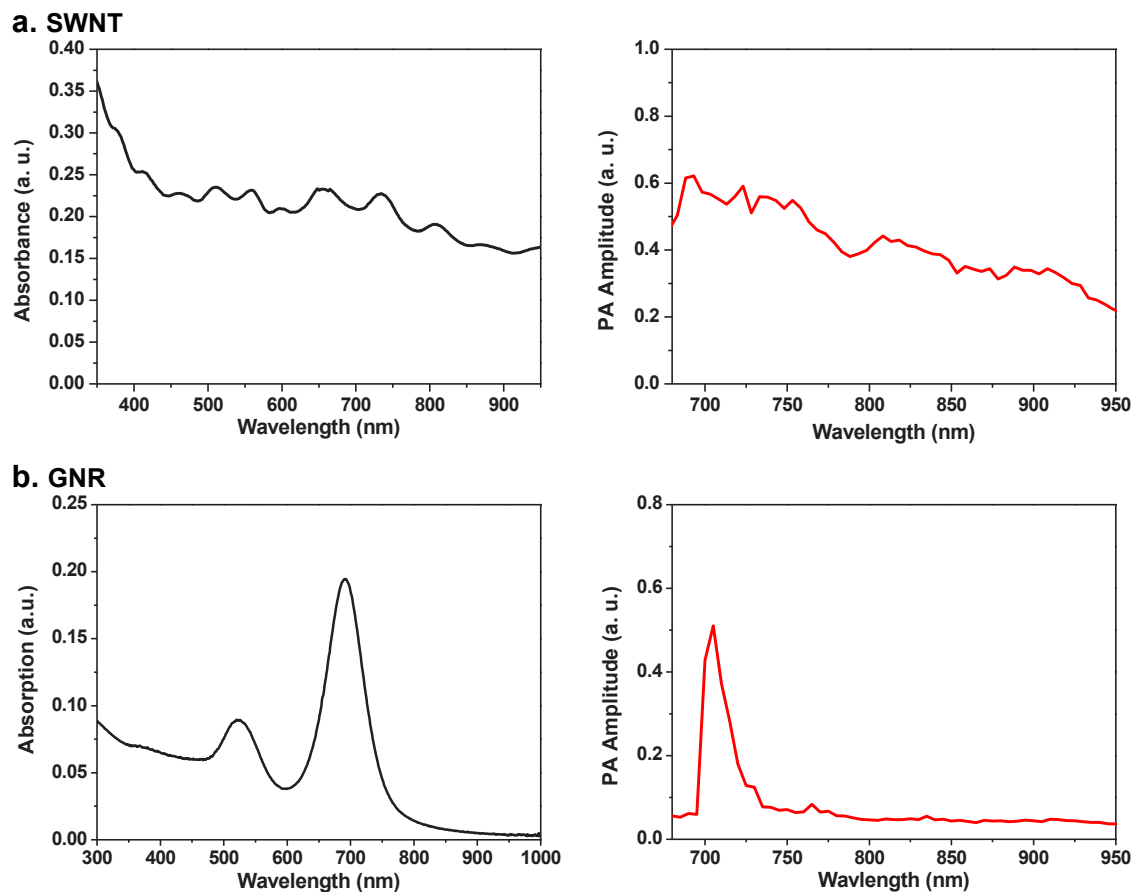
Supplementary Fig. S2: (a) Fluorescence spectrum of SPN1 in 1×PBS (pH = 7.4) upon excitation at 680 nm. (b) Fluorescence images of the SPN1 solutions (pH = 7.4) at different concentrations. Images were obtained from IVIS with excitation at 680 nm and emission at 840 nm.



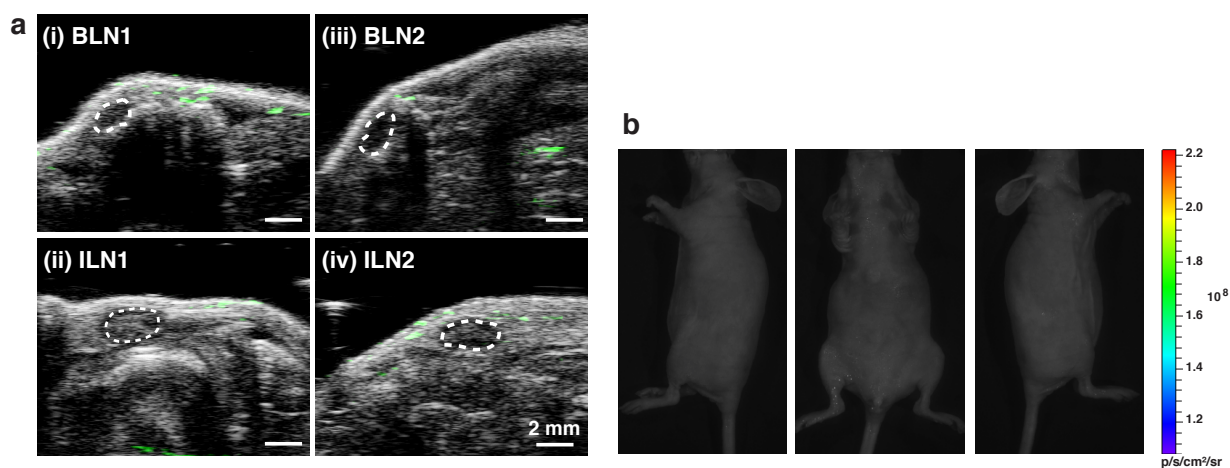
Supplementary Fig. S3: (a) Schematic of the tissue-mimicking phantom containing hemoglobin and intralipid implanted with two polyethylene (PE) tubes filled with SPN1 at a concentration of 40 (Tube 1) and 20 (Tube 2) $\mu\text{g/mL}$, respectively. (b) Fluorescence/bright-field overlaid top-view image of the tissue-mimicking phantom. (c) 3D PA/US coregistered image of the tissue-mimicking phantom. (d) 2D PA top-view image of the tissue-mimicking phantom. PA imaging clearly visualized both tubes with nearly no signal attenuation up to a depth of 1 cm. However, neither tube was distinguished by FL imaging, with significant signal loss within 0.5 cm of imaging depth.



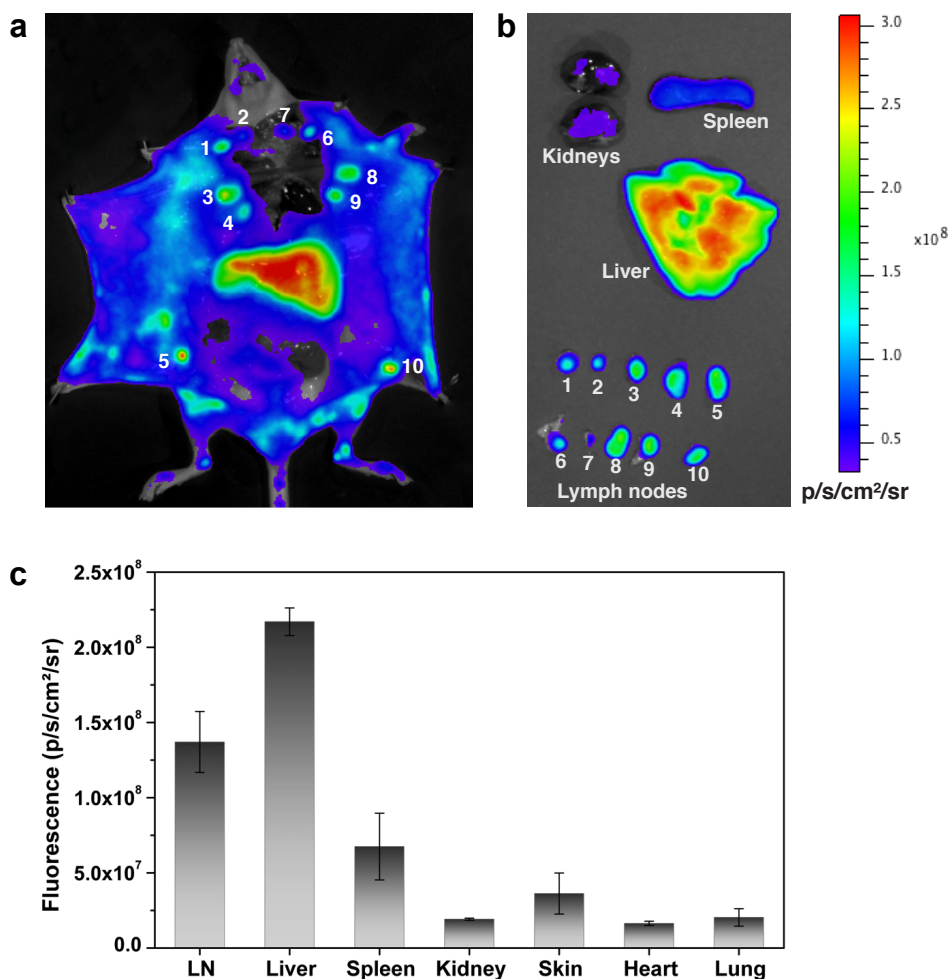
Supplementary Fig. S4: (a) Fluorescence/bright-field overlaid image of a PE tube filled with SPN1. (b) Fluorescence/bright-field overlaid image of a mouse with the SPN1-filled PE tube implanted subcutaneously at the dorsal aspect of the leg. Red dash line encircles the location of the tube. (c) US (top) and PA/US coregistered (bottom) images of the SPN1-filled PE tube in an agar phantom. White arrows indicate the sidewalls of tubes. [SPN1] = 20 µg/mL. (d) PA (top) and PA/US coregistered (bottom) images of the mouse leg subcutaneously implanted with tubes filled with the nanoparticle solution (left) or water (right), respectively. White arrows indicate the sidewalls of tubes. [SPN1] = 20 µg/mL. Only PA signals at the walls of the tube are visible because only the higher frequency components of the PA generated pressure wave are detected. PA imaging clearly delineated the SPN1-filled PE tube through overlying muscle, skin, and connective tissue of the intact thigh of a living mouse, but no signal was detectable for FL imaging. Moreover, the inner diameter of the tube was measured to be 0.70 ± 0.02 mm from the PA images both *in vitro* and *in vivo*, which matched well with its actual value (0.70 mm).



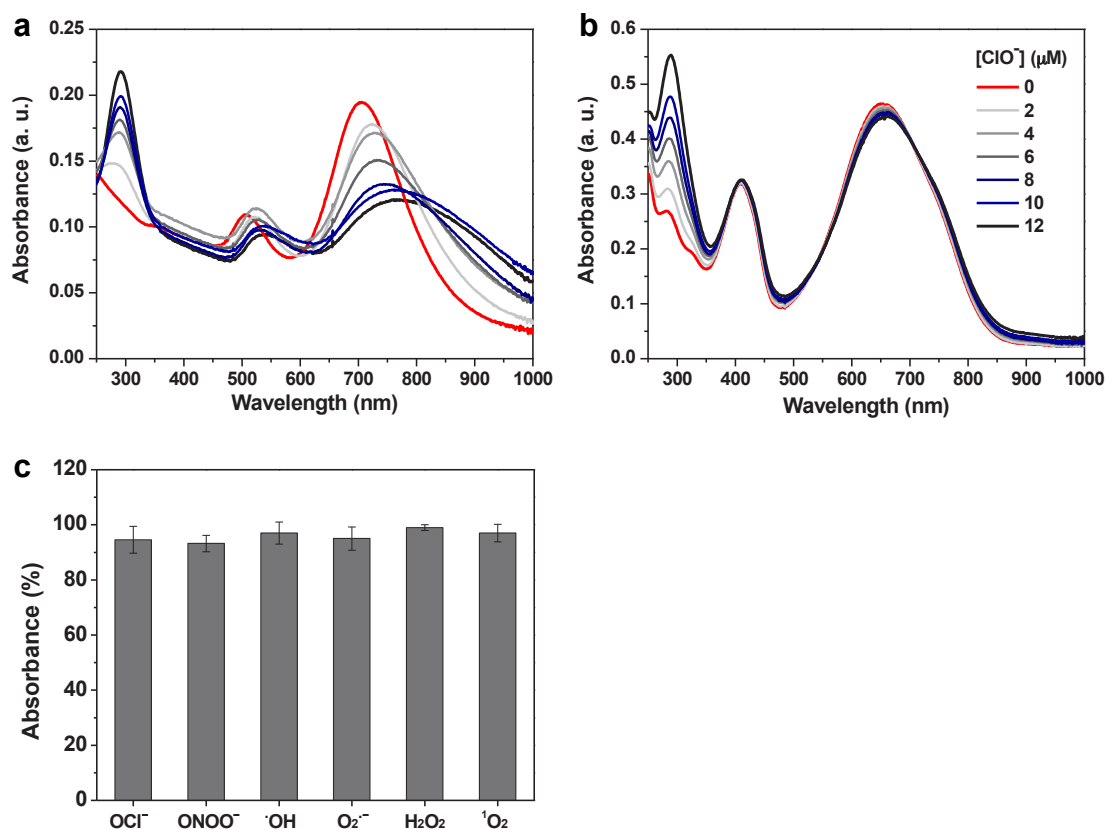
Supplementary Fig. S5: UV-Vis (left) and PA (right) spectra of SWNT (a) and GNR (b). SWNT: 1.2×150 nm; GNR: 15×40 nm. [SWNT] = [GNR] = 5 $\mu\text{g/mL}$.



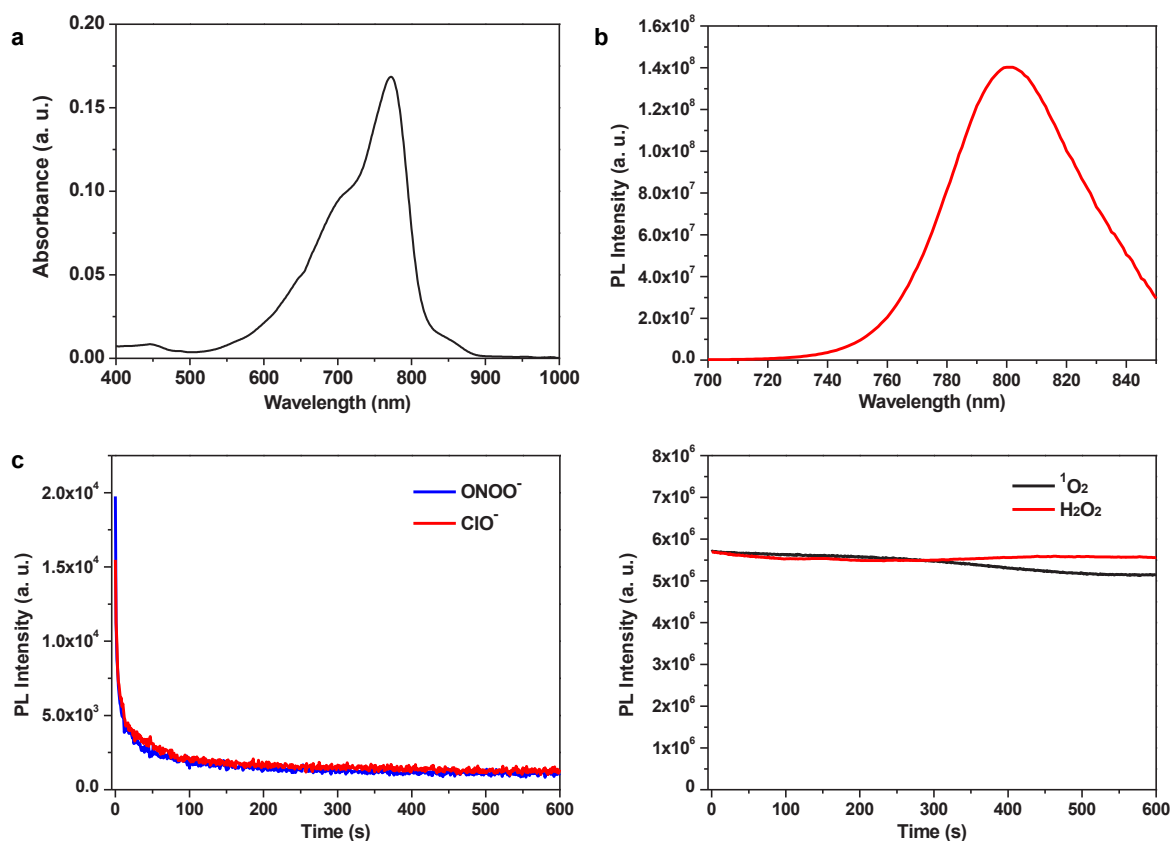
Supplementary Fig. S6: PA/US co-registered (a) and FL/bright-field (b) images of a representative mouse before tail vein injection of SPN1.



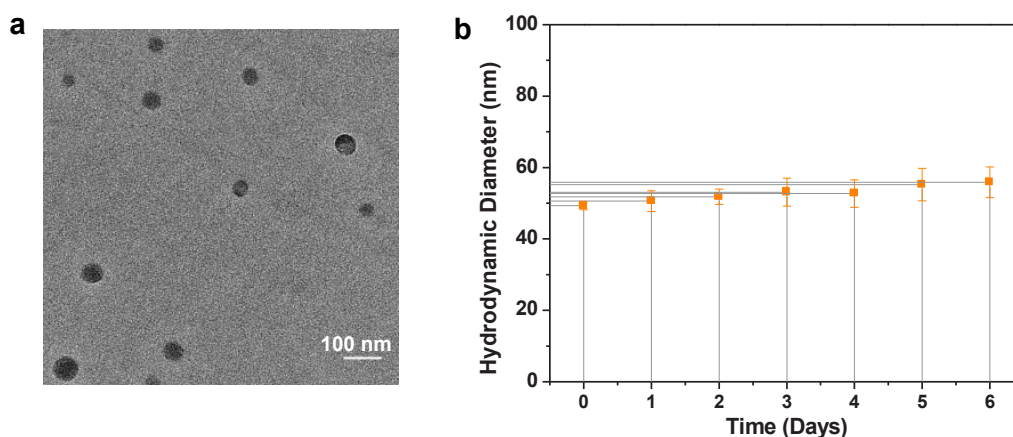
Supplementary Fig. S7: (a) Fluorescence/bright-field image of a mouse administered SPN1 (50 μg) after necropsy and skin resection to expose the intact peritoneal cavity, lymph nodes, and milk line of the mouse. 1, 2, 6&7 are superficial cervical lymph nodes (SCLNs), 3&8 are axillary lymph nodes (ALNs), 4&9 are brachial lymph nodes (BLNs), and 5&10 are inguinal lymph nodes (ILNs). (b) Fluorescence/bright-field image of the organs from the corresponding mouse. (c) Quantification of the fluorescence signals of the organs from mice (n=4) administered SPN1 (50 μg) *via* tail vein injection. Error bars represent standard deviation of four separate measurements.



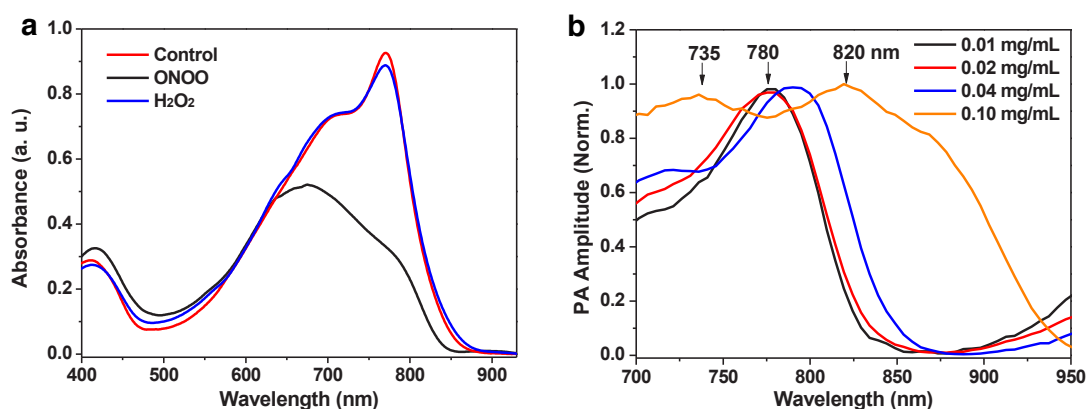
Supplementary Fig. S8: ROS stability study of SPN1 and GNR. UV-Vis spectra of GNR (**a**) and SPN1 (**b**) in the absence or presence of ClO^- at concentrations ranging from 0 to 12 μM at an interval of 2 μM . (**c**) Changes in the absorption of SPN1 at 700 nm after incubation with ROS for 15 min. $[\text{SPN1}] = [\text{GNR}] = 5 \mu\text{g/mL}$; $[\text{ROS}] = 12 \mu\text{M}$. The data show that SPN1 is highly resistant to ROS. Error bars represent standard deviation of three measurements.



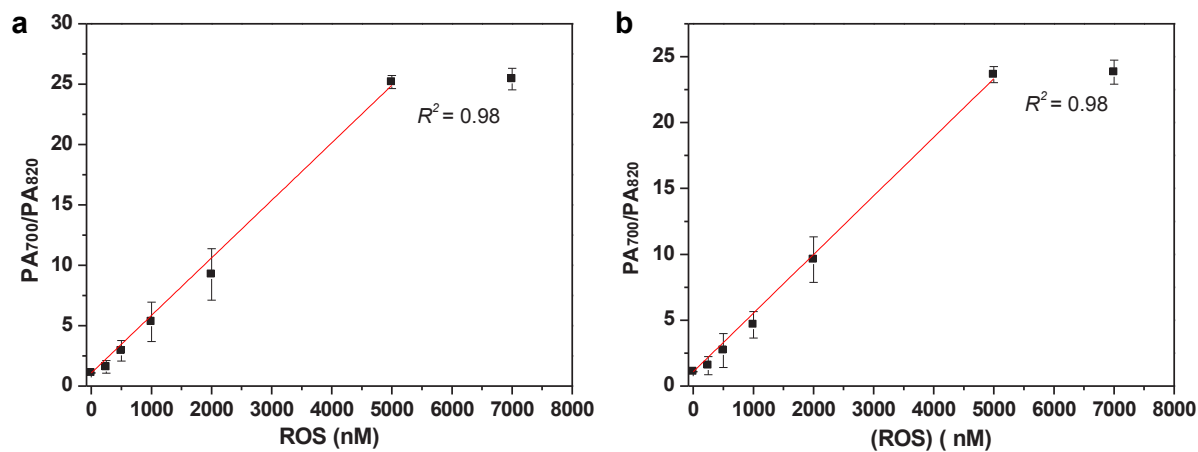
Supplementary Fig. S9: Kinetic study of ROS-mediated degradation of IR775S by detecting its fluorescence decrease at 800 nm after addition of ROS. UV-vis absorption (a) fluorescence (b) spectra of IR775S. (c) Time course of the fluorescence of IR775S at 800 nm after addition of indicated ROS (such as ONOO^- , ClO^- , $^1\text{O}_2$ and H_2O_2) in $1 \times \text{PBS}$ ($\text{pH} = 7.4$). $[\text{IR775S}] = 1 \mu\text{g/mL}$. $[\text{ROS}] = 2 \mu\text{M}$. Dashed line (control) shows the intensity level of the intact IR775S. The rapidly decreased fluorescence indicates that IR775S undergoes nearly instant degradation in the presence of peroxynitrite (ONOO^-) and hypochlorite (ClO^-).



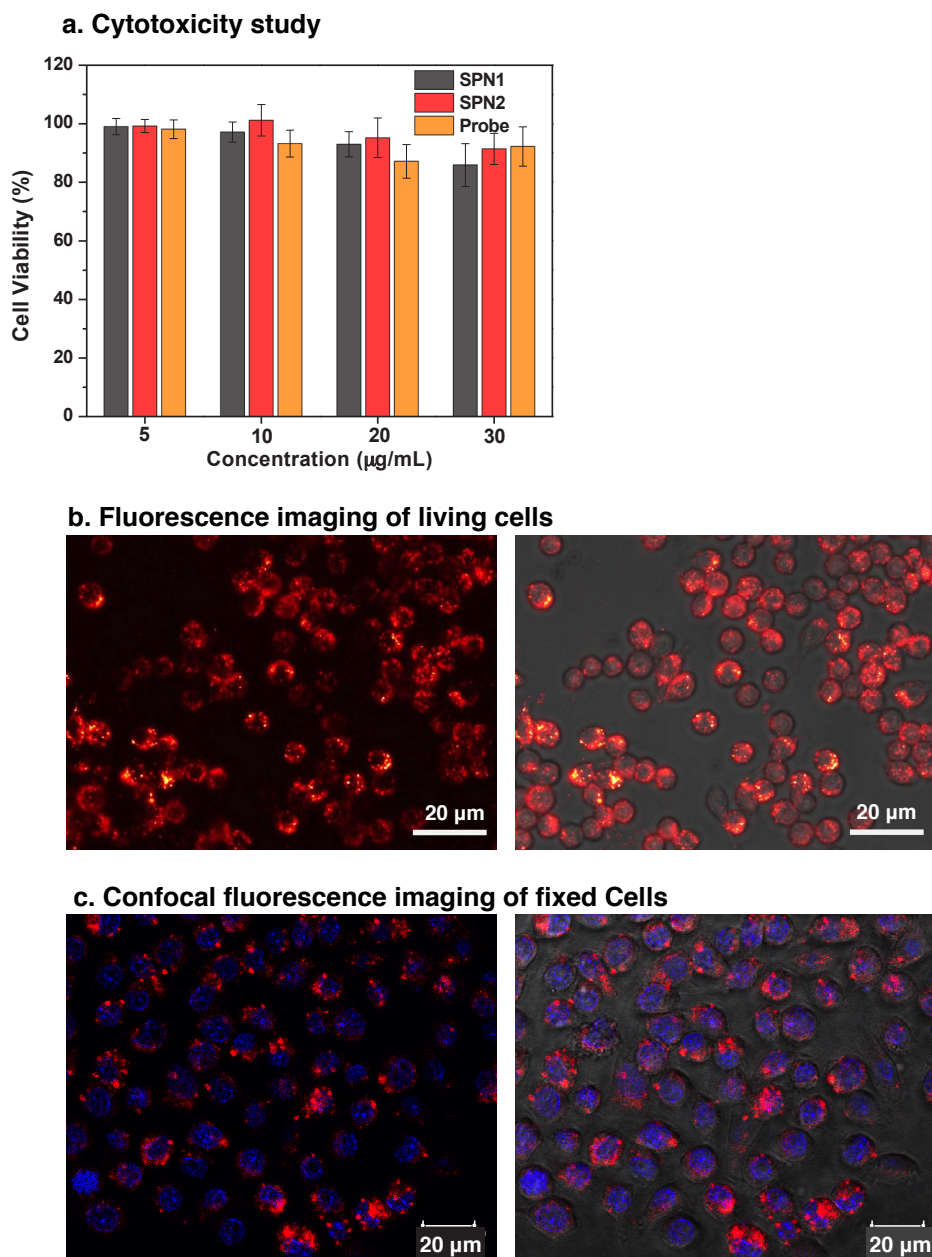
Supplementary Fig. S10: (a) Representative TEM of RSPN. (b) Size stability study of RSPN in 1×PBS (pH = 7.4) over time. Error bars represent standard deviation of three separate measurements.



Supplementary Fig. S11: (a) Representative UV-Vis spectra of RSPN in the absence (red) and presence (blue = hydrogen peroxide, black = peroxynitrite) of ROS. [RSPN] = 5 $\mu\text{g mL}^{-1}$ and [ROS] = 5 μM . (b) Normalized PA spectra of IR775S at different concentrations in agar phantom. The mass concentration of 0.10 mg/mL is equal to the molar concentration of 0.20 mM. The PA spectrum of IR775S at a low concentration (0.01–0.04 mg/mL) (Fig. S11b) is similar to its absorption spectrum (Supplementary Fig. S9a) with nearly the same maximum at 780 nm. However, when the dye concentration increases to 0.1 mg/mL, dye aggregates formed and its PA spectrum changes with two new peaks (735 and 820 nm).



Supplementary Fig. S12: PA_{700}/PA_{820} of RSPN (5 $\mu\text{g/mL}$) as a function of the concentrations of ONOO^- (a) and ClO^- (b). The PA ratio shows a linear correlation with the concentration of both ROS. The saturation occurs at $[\text{ROS}] = 5 \mu\text{M}$. Error bars represent standard deviation of three separate measurements.



Supplementary Fig. S13: (a) *In vitro* viability of RAW264.7 cells treated with SPN solutions at concentrations of 5, 10, 20, or 30 µg/mL for 24 h. The percentage cell viability of treated cells is calculated relative to that of untreated cells with control viability defined as 100%. Error bars represent standard deviation of three separate measurements. (b) Fluorescence (left) and fluorescence/bright-field overlay (right) images of live macrophage RAW 264.7 cells after incubation with SPN probe (6 µg/mL) for 3 h. (c) Confocal fluorescence (left) and fluorescence/bright-field overlaid (right) images of fixed macrophage RAW 264.7 cells after incubation with SPN probe (6 µg/mL) for 3 h. Cell nucleus was stained by DAPI.

2. Supplementary Methods

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-PEG2000 (PL-PEG2000) were purchased from Avanti Lipids. Poly[cyclopentadithiophene-*alt*-benzothiadiazole] (SP1) ($M_w = 20872$) was purchased from Sigma-Aldrich.

2.2. Materials Characterization

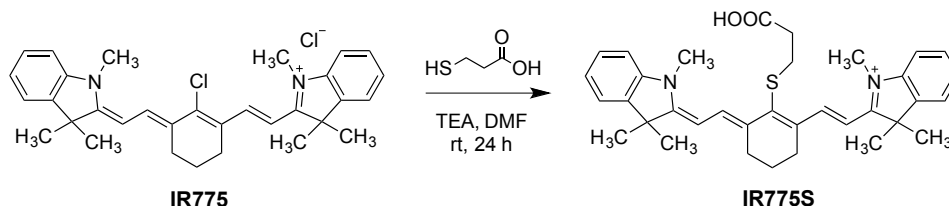
NMR spectra were taken on Bruker 400 MHz magnetic resonance spectrometer. ESI-MS was performed at the Mass Spectrometry Facility of Stanford University. HPLC was performed on a Dionex HPLC System (Dionex Corporation) equipped with a GP50 gradient pump and an inline diode array UV-Vis detector. TEM images were obtained on a JEM 1230 transmission electron microscope with an accelerating voltage of 200 kV. Dynamic light scattering (DLS) was performed on a 90 plus particle size analyzer (Brookhaven Instruments Corporation). Zeta potential measurements were conducted on the Malvern ZetaSizer Nano S. UV-vis spectra were recorded on an Agilent spectrophotometer. Fluorescence measurements were carried out on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon). Fluorescence living cell and confocal fixed cell imaging were conducted on Olympus inverted fluorescence microscope (IX2-UCB) equipped with a Nuance multispectral imaging camera and Leica SP5 confocal upright microscope system (DM6000), respectively. Fluorescence animal imaging was acquired with xenograft IVIS spectrum imaging system. The quantum yield of SPNs was measured using indocyanine green (ICG) as the standard with known a quantum yield of 1% in H₂O.

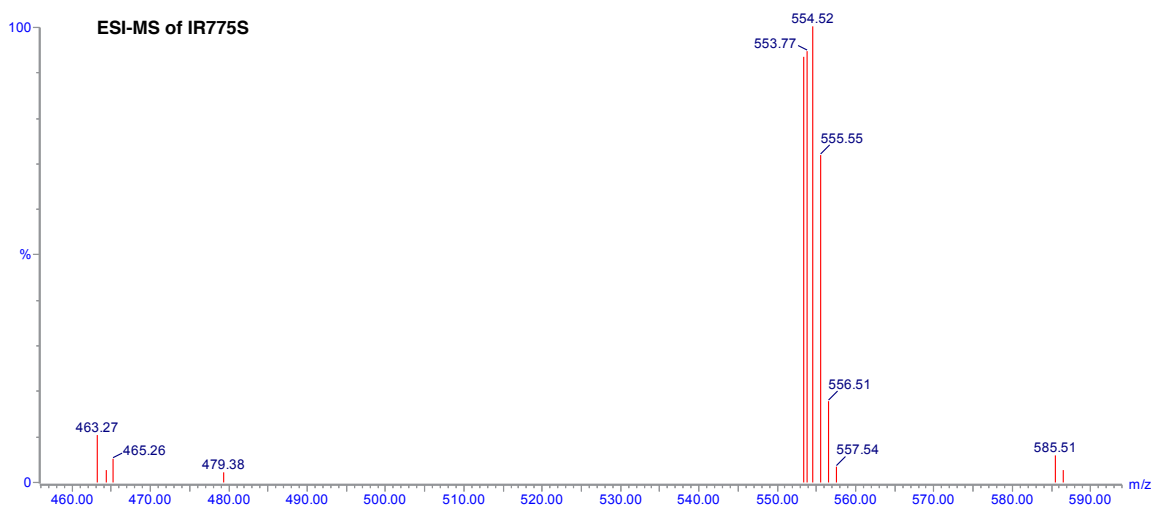
2.3. PA Instrumentation

PA spectra and PA/US coregistered images were acquired with a LAZR commercial instrument (Visualsonics, 2100 High-Resolution Imaging System). It is equipped with a MS-250 linear array transducer (21 MHz, 70%-6 dB two-way bandwidth, 256 elements) to detect US and PA signals, and a tunable Nd:YAG laser system (OPOTEK Inc., Carlsbad, CA, 680-950 nm, 20 Hz repetition rate, 5 ns pulse width, 50 mJ pulse peak energy) was used to trigger the system acquisition, and excite tissue with optical pulses to generate the PA effect. The spot size is 1×24 mm, and the full field of view is 14–23 mm wide. According to the information provided by manufacturer, the axial and lateral resolution limits for PA and US images are 75 and 165 μm , respectively. Acquisition rate of 5 frames per second was used for all the experiments.

2.4. Synthesis of IR775S¹

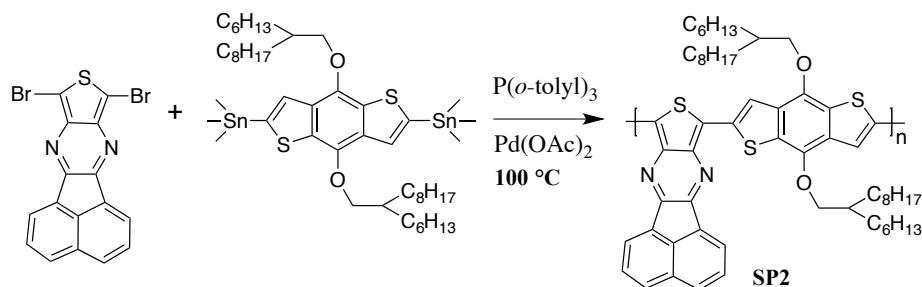
To a solution of IR775 chloride (200 mg, 0.39 mmol) in DMF (1 mL) were added 3-mercaptopropionic acid (40 μL , 0.44 mmol) and triethylamine (85 μL , 0.59 mmol). After stirring at room temperature for 24 hours, then CH_2Cl_2 was added to the reaction mixture, and the whole was washed with brine. The organic extracts were dried over Na_2SO_4 , filtered, and evaporated. The crude product was purified by HPLC (125 mg, yield 58%). ^1H NMR (400 MHz, CD_3OD): δ : 8.87 (d, $J = 14.3$ Hz, 2H), 7.54 – 7.47 (m, 2H), 7.41 (m, 2H), 7.27 (m, 4H), 6.27 (d, $J = 14.2$ Hz, 2H), 3.66 (br, 6H), 3.07 (t, $J = 6.8$ Hz, 2H), 2.69 (t, $J = 6.0$ Hz, 4H), 2.59 (t, $J = 6.8$ Hz, 2H), 2.03 – 1.87 (m, 2H), 1.74 (s, 12H). ESI-MS: m/z calcd. $[\text{M}]^+$: 553.29; found, $[\text{M}]^+$: 553.77.





2.5. Synthesis of SP2

The polymer was prepared according to the previous literature.² Briefly, equal mole of 8,10-dibromoacenaphtho[1,2-e]thieno-[3,4-b]pyrazine and (4,8-bis((2-hexyldecyl)oxy)benzo[1,2-*b*:4,5-*b'*]dithiophene-2,6-diyl)bis(trimethylstannane) was dissolved into dichlorobenzene and was purged with argon for 20 min, followed by the addition of tri(*o*-tolyl)phosphine (5 mol%) and tris[dibenzylideneacetone]dipalladium(0) (2.5 mol%). The resulting solution was heated to 100 °C. Crude polymer was precipitated in methanol, and then subjected to Soxhlet extraction for further purification. ¹H NMR (400 MHz, CDCl₃): δ 0.5–2.2 (m, 62 H), 4.1–4.6 (m, 4H), 3.1 (br, 4H), 7.2–8.5 (m, 8H). GPC (THF): M_w = 174,354.



2.6. Preparation of SPNs

A THF solution of SP1 or SP2 (0.25 mg/mL, 1 mL) was rapidly injected into distilled-deionized water (9 mL) under continuous sonication with a microtip-equipped probe sonicator (Branson, W-150) at a power output of 6 watts RMS for 30 S. Then, DPPC (0.25 mg/mL, 1 mL) in THF/water (4: 6) was added into the mixture. After sonication for additional 1 min, THF was evaporated at 45 °C under nitrogen atmosphere. The aqueous solution was filtered through a polyethersulfone (PES) syringe driven filter (0.22 µm) (Millipore), and washed three times using a 30 K centrifugal filter units (Millipore) under centrifugation at 4,000 rpm for 3 min at 4 °C. The nanoparticle solution was finally concentrated to 0.08 mg mL⁻¹ (based on the mass of SP) by ultrafiltration and stored in dark at 8 °C.

2.7. Preparation of RSPN

A THF mixture (1 mL) containing SP1 (0.25 mg/mL) and IR775S (0.05 mg/mL) was rapidly injected into deoxygenized distilled-deionized water (9 mL) under continuous sonication with a microtip-equipped probe sonicator (Branson, W-150) at a power output of 6 watts RMS for 30 S. Then, DPPC (0.5 mg/mL, 1 mL) in THF/water (4: 6) was added into the mixture. After sonication for additional 1 min, THF was evaporated at 45 °C under nitrogen atmosphere. The aqueous solution was filtered through a polyethersulfone (PES) syringe driven filter (0.22 µm) (Millipore), and washed three times with deoxygenized water using a 30 K centrifugal filter units (Millipore) under centrifugation at 4,000 rpm for 3 min at 4 °C. The nanoprobe solution was finally concentrated to 0.08 mg/mL (based on the mass of SP1) by ultrafiltration and stored in dark under nitrogen atmosphere at 8 °C.

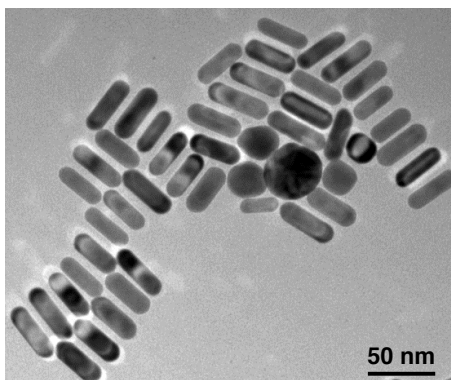
2.8. Synthesis of SWNTs and GNRs

The synthesis of phospholipid-PEG functionalized SWNTs (150 nm in average length and 1.2 nm in average diameter) was synthesized according to our previous report.³ Raw Hipco

SWNTs were sonicated in an aqueous solution of PL–PEG2000 for 1 h, centrifuged at 24,000 g for 6 h to obtain short, phospholipid-PEG-functionalized SWNTs in supernatant. Filtration through 100-kDa filters (Millipore) removed excess phospholipids. The average molecular weight is 170 kDa per single-walled carbon nanotube.

The PEG functionalized GNRs (15×40 or 12.0×41 nm on average) was synthesized according to our previous report.⁴ The GNRs were prepared *via* the seeded-growth mechanism previously described with slight modifications. Briefly, gold seed was prepared by the addition of 5 mL of 0.2 M CTAB to 5 mL of 0.005 M gold chloride in a scintillation vial. Then, 0.6 mL of 0.01 M NaBH₄ (previously chilled for 10 min in an ice water bath) was quickly added, and the mixture was shaken for 2 min. The growth mixture was prepared with the following: 250 mL of 0.2 M CTAB, 250 mL of 0.001 M AuCl₃, and 12 mL of 4 mM AgNO₃. This solution was yellow/brown, but became translucent upon the addition of 3.5 mL of 0.0788 M ascorbic acid. Seed (0.6 mL) was then added, and the solution became purple/brownish over 30 min. The GNRs were purified with four rounds of centrifugation and water washing at 16 000 rcf for 20 min. To remove CTAB and coat the GNRs with PEG, ligand exchange was performed by dialyzing the GNRs with thiol-PEG *vs* distilled water. The sizes of GNRs were determined by DLS, and their morphologies were confirmed by TEM. The representative TEM of GNRs are shown as follows:

TEM of GNR



2.9. PA detection of ROS

H_2O_2 , ONOO^- , OCl , and $\text{O}_2^{\bullet-}$, stock solutions were prepared by directly diluting commercially available H_2O_2 , NaONOO , NaOCl , and KO_2 , respectively. NO was generated from diethylamine NONOate. $\cdot\text{OH}$ was generated from Fenton reaction between H_2O_2 and $\text{Fe}(\text{ClO}_4)_2$. $^1\text{O}_2$ was produced from the H_2O_2 /molybdate ions (Na_2MoO_4) system. RSPN (5 $\mu\text{g/mL}$) was incubated with ROS (5 μM) for 1 min in 1 $\times$ PBS (pH = 7.4). For kinetic studies, the fluorescence intensities of IR775S were measured at intervals of 1 s immediately after addition of ROS. PBS used for these experiments was purged with N_2 for 1 h before the measurement. Note that the reaction between ROS and RSPN is nearly instant (within 30 s), and mainly constrained by the methods used to generate ROS. PBS used for these experiments was purged with N_2 for 1 h before the incubation. To each testing solution (30 μL) was added a warm agar solution (1%, 20 μL , 40 $^\circ\text{C}$), and the resulting mixture was added to agar phantom well. The phantom was sealed a final ~ 4 mm of agar. Typical PA measurement conditions include 100% power, 40 dB gain, 21 MHz frequency, and spectral acquisition from 680 to 950 nm with a 1 nm step size.

2.10. Cell culture

RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (ATCC). RAW 264.7 cells were cultured in DMEM (Dulbecco's Modified Eagle

Medium) (GIBCO) supplemented with 10% FBS (fetal bovine serum) (GIBCO). The cell line was maintained in an atmosphere of 5% CO₂ and 95% humidified air at 37 °C.

2.11. Cytotoxicity test

Cells were seeded in 96 well plates (1000 cells in 100 µL per well) for 24 h, and then SPNs (final concentration 5, 10, 20, and 30 µg/mL) was added to the cell culture medium. Cells were incubated with or without (control) SPNs for 24 h, followed by the addition of MTT (20 µL, 5 mg/mL) for 3 h. The media was removed and DMSO (200 µL) was added into each well and gently shaken for 10 min at room temperature to dissolve all formed precipitate. The absorbance of MTT at 550 nm was measured by using a microplate reader. Cell viability was expressed by the ratio of the absorbance of cells incubated with SPN solution to that of cells incubated with culture medium only.

2.12. Fluorescence live and fixed cell imaging

RAW 264.7 cells were seeded into 8-well chamber slides and allowed to adhere for 18 h in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The cells were treated with SPNs (final 6 µg/mL) for 3 h in FBS-free culture medium. Cells were washed three times with 1 × PBS prior to living cell imaging. Cy5 channel was used to excite the cells and fluorescence signals were collected at 725 ± 50 nm. Cells were also grown on sterile glass slides, treated with SPNs under the same conditions, and then fixed with 4% paraformaldehyde (PFA) and stained with 4',6-diamidino-2-phenylindole (DAPI) for fluorescence confocal imaging with excitation at 594 nm and signal collection from 650 to 800 nm.

2.13. PA detection of endogenous ROS of RAW 264.7

RAW 264.7 cells were seeded into cell culture flask (250 mL) and allowed to adhere for 18 h in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. LPS (final 1 µg/mL) and IFN-

γ (final 50 ng/mL) was added and incubated for 4 h. The cells were then treated with RSPN (final 6 μ g/mL) for 3 h in FBS-free culture medium. Alternatively, the cells were pretreated with NAC (final 1 mM) 2 h before LPS (final 1 μ g/mL) and IFN- γ (final 50 ng/mL) treatment. The cells were treated with NAC (final 1 mM) for 1 h again before incubation with RSPN (final 6 μ g/mL) for 3 h. Cells were washed three times with 1 $\times$ PBS buffer prior to trypsinization. The cell suspension (5×10^6) was concentrated by centrifugation, and suspended in warm water (50 μ L, 40 °C). To the suspension was added the warm agar solution (1%, 50 μ L, 40 °C), and the resulting mixture was maintained in liquid form at 40 °C until its use in an agar phantom. The agar cell suspensions (30 μ L) were added to each agar phantom well for PA imaging.

2.14. Preparation of tissue phantom⁵

Tris-buffered saline was poured on top of a known amount of sodium azide and gelatin to make a final concentration of 15 mM and 10 w/v%, respectively. The mixture was heated to 50 °C using a water bath under constant stirring. After the gelatin had dissolved, the mixture was cooled to 37 °C with constant stirring. Hemoglobin and intralipid were added to reach concentrations of 50 μ M and 1 v/v%, respectively. A gel cassette intended for electrophoresis was used as a mold to form a 1 cm thick tissue phantom embedded with two SPN1-filling tubes (Tube 1: 40 μ g/mL; Tube 2: 20 μ g/mL) as shown in **Figure S3a**. The absorption coefficient (m_a) and the scattering coefficient (m_s) at 700 nm were extrapolated from the literature to be approximately 1.0 mm⁻¹ and 0.5 mm⁻¹, respectively.⁵ As such, the gel phantom is a relevant physiological tissue mimic for muscle and represents a good physiological model for *in vivo* tissue.

2.15. Imaging of tube phantom *in vivo*

Tube phantoms were constructed from ~2 cm lengths of polyethylene (PE) tubing with inner diameters of 0.7 mm. The tubes were heat-sealed at one end, filled with either water or SPN1, and then heat-sealed on the opposing side. Female nu/nu mice (8 weeks old, Charles River Inc.) were anesthetized, and two 2 mm long incisions were made at the superior and inferior edges of the dorsal aspect of the right upper hind limb. The PE tubes were cleaned with isopropanol and were then inserted through the incisions, placing them between the skin and muscle of the upper hind limb. Mice were positioned so that PA imaging could be performed on the ventral aspect of the lower right leg using a Vevo LAZR with excitation at 700 nm.

2.16. Imaging nanoparticle solutions in matrigel *in vivo*

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by the Stanford University Animal Studies Committee. In order to compare the *in vivo* signal evolution at equal mass concentrations of SPN1, GNR, and SWNT, nanoparticles were suspended in Matrigel (BD Biosciences) to different concentrations. The final concentration of Matrigel was 50% v/v for all solutions. Matrigel-nanoparticle mixtures (30 mL) were injected subcutaneously on the dorsal aspects of female nude mice (Charles River Inc.) to form inclusions of nanoparticles, and PA imaging was performed using a Vevo LAZR imaging system with excitation wavelength of 700 nm. Three separate inclusions of each concentration, as well as of Matrigel alone, were imaged.

2.17. Lymph node PA imaging

Female nu/nu mice (8 weeks old, Charles River Inc.) received either SPN1 (50 µg in saline) in sterile saline (n=4) or sterile saline (n=4) intravenously, and were returned to their cages. Mice were then anesthetized by isoflurane 24 h later in preparation for imaging. PA imaging was first performed using the Vevo LAZR system with 100% power, 40 dB gain, 21

MHz frequency, and excitation of 680 nm. Mice were positioned on a heated platform so that the general location of the lymph node of interest was directly facing the transducer. Lymph nodes were identified as hypoechoic ovals within the hyperechoic fat pads of mice, just inferior to the overlying epidermal-dermal layer. Some lymph nodes (especially cervical) showed a hyperechoic hilum below the hypoechoic cortex, which is a sonographic trademark of lymph nodes.⁶ The lack of peristalsis differentiated the inguinal lymph nodes from bowel, which may also be hypoechoic and oval in shape. Following PA imaging, mice were placed in an IVIS Spectrum for *in vivo* fluorescence imaging, with excitation of 680 nm and emission of 840 nm. Images were acquired ventrally, and from both the right and left lateral aspects. After imaging, mice were sacrificed by CO₂ asphyxiation, and necropsy was performed by a midline incision ventrally from caudal to rostral aspects of the mouse through both the skin and peritoneum. The skin was pinned so as to expose the lymph nodes prior to fluorescence imaging using the same parameters described above. Organs were subsequently removed, rinsed in saline, and a fluorescence image was acquired immediately with an IVIS Spectrum using the same parameters listed above. For *ex vivo* imaging, lymph nodes were embedded in agar phantom and acquired using the Vevo LAZR system with 100% power, 40 dB gain, 21 MHz frequency, and excitation at 680 nm.

2.18. Edema mouse model

In order to induce an inflammatory-like syndrome in mice, a mouse model of zymosan-induced local hind limb edema was employed as previously reported.⁷ Briefly, zymosan (2 mg in 100 μ L sterile saline) was injected intramuscularly into the right biceps femoris muscle of the upper hind limb (n=3). Following an incubation period of 20 min, RSPN (3 μ g in 50 μ L saline) were injected at the same location in the upper hind limb, and PA signals were recorded using a

Vevo LAZR system in Nanostepper mode with alternating acquisition at 680 nm and 820 nm over the volume of the thigh. For a control, non-inflammatory condition (n=3), sterile saline (100 μ L) was injected into the biceps femoris of the left upper hind limb, and the same procedure described above was followed.

2.19. Data Analysis

PA signal intensities were measured by region of interest (ROI) analysis using the Vevo LAZR imaging system software package. The PA signals reported were the average pixel intensities from within the ROI. The pixel intensities are proportional to the pressure generated by the PA effect and were representative of pressure amplitudes. For ratiometric image processing, raw images were exported to Image J software for pseudo color analysis and image overlay using standard software procedures. Results are expressed as the mean $\pm$ standard deviation unless otherwise stated. Statistical comparisons between two groups were determined by t-test, and between 3 or more groups by one-way ANOVA followed by a post-hoc Tukey's HSD test. Time course analysis between groups was performed by general linear model repeated-measures analysis. For all tests, $p < 0.05$ was considered statistically significant. All statistical calculations were performed using GraphPad Prism v. 5 (GraphPad Software Inc., CA, USA).

3. Supplementary Discussion

The maximum PA signals were observed at 690 and 705 nm for SPN1 and SPN2, respectively, both of which were red-shifted as compared to their maximum absorption (**Fig. 1f**). Two possible factors may contribute to this phenomenon. Firstly, π -conjugated polymer chains are highly compressed within SPNs, leading to heterogeneous π - π stacking,^{8,9} the formation of

weakly emissive ground-state aggregates and different thermal deactivation efficiencies among polymer segments.¹⁰ Secondly, optical illumination parameters used for PA measurements (high power pulsed laser) and absorption spectroscopy measurements (low power continuous wave illumination) differ, where high-power pulsed lasers can produce certain types of wavelength-dependent short-lived photosaturation effects.¹¹

4. References

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